UPON AN ANTI-CHOLERA SERUM

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It is generally accepted that the symptoms of cholera Asiatica are the result of an acute intoxication with certain products of the specific agent—the comma bacillus of Koch. Further, that these symptoms are due not to a general invasion of the body by the bacillus but to an absorption of its toxins from the seat of infection—the intestine. An intoxication being the cardinal feature in the disease the character of the specific poison became naturally a subject of close investigation, and more than one attempt has been made to isolate it and to bring the treatment of cholera within the range of serum therapeutics. Little hope of success has been found to lie in the preparation and the use of a bactericidal serum. The serum therapy of cholera, if it is to meet with any success, must rest on an antitoxic basis, bearing in mind the clinical features of the disease. This also has generally been recognised by those who have endeavoured to introduce specific methods of treatment.

Numerous efforts have been made to obtain toxins from cultures of the cholera organism. Two theories, apparently contradictory, arose with regard to the nature of the specific toxins and gave rise to much controversy. It has been maintained, notably by French observers, that the poison is a genuine secretory product, both *in vita* and *in vitro*, and extracellular in the sense of being diffusible and soluble. Others, and pre-eminently German observers, maintain that the poison is inherent to the cell plasma and therefore of the intracellular type. The point of view determined in each case the method of investigation employed. The upholders of an extracellular poison endeavoured to obtain a secreted toxin by the cultivation of the bacillus on fluid laboratory soils, whilst those who held the poison to be intracellular worked with the killed and undoubtedly toxic bodies of the bacilli. It may be useful to summarise the main results that were obtained inasmuch as a good deal of the work dates ten years back.

Among the earlier observers Petri[1] grew the comma bacillus on peptone soils and obtained a heat-resistant proteid termed by him "toxo-peptone." The entire cultures,

[1] Petri: Arbeiten aus dem Kaiserlichen Gesundheitsamte, Band vi., 1890.

sterilised by heat, proved to be more toxic than their filtrates. Hueppe and Scholl [2] cultivated the bacilli in fresh sterile eggs. The alcoholic precipitate from the egg yielded an aqueous extract poisonous to the guinea-pig. This effect was, however, shown to be due mainly to ordinary decomposition products and the retained alcohol. Gamaleia,[3] as the result of his investigations, came to the conclusion that there are several cholera toxins. The organisms grown on a special veal broth yielded two soluble poisons—the one, thermolabile, produced diarrhœa in the rabbit, and the other, thermostable, caused an intoxication but without diarrhœa. The doses required to bring about these effects were considerable. Behring and Ransom in 1895 [4] described a soluble poison obtained by them from fluid cultures of the comma bacillus which was heat-resistant and acutely toxic to the guinea-pig in concentrated doses. For this poison they were able to prepare an antitoxin. It can hardly be supposed that these observers were dealing so much with the genuine poison as with secondary products which are to be met with in old cultures of the cholera and other organisms. Nor does their serum appear to have exhibited antitoxic properties much more striking than those of the normal serum. The important researches of Metchnikoff, Roux, and Salimbeni [5] call for detailed notice, both on account of the ingenious methods employed and the results obtained. Highly virulent cultures of the comma bacillus, prepared by cultivation in collodion sacs in the peritoneal cavity of the guinea-pig, were sown on a special medium containing 2 per cent. peptone, 2 per cent. gelatin, and 1 per cent. sodic chloride. A short period of incubation was given to the cultures in order to obtain the toxin before it had undergone modification. The cultures reached their maximum toxicity on the fourth day and were then filtered. The filtrate was toxic on subcutaneous injection in doses of 0·3 cubic centimetre per 100 grammes weight of guinea-pig. This toxin, similarly to Behring and Ransom's, was not sensibly modified at boiling point but lost its activity under the action of air and light. The guinea-pig was most susceptible to the toxin, other animals, such as the rabbit, requiring larger doses. The poison acted subcutaneously and peritoneally. The effects consisted in hypothermia, peritonitis, fluid distension of the small

[2] Hueppe: Deutsche Medicinische Wochenschrift, p. 417, 1891.
[3] Gamaleia: Archives de Médecine Expérimentale, p. 172, 1892.
[4] Behring: Deutsche Medicinische Wochenschrift, p. 294, 1898; Ransom: ibid., p. 457, 1895.
[5] Metchnikoff, Roux, and Salimbeni: Annales de l'Institut Pasteur, vol. x., p. 257, 1896.

intestine, along with hyperæmia of the abdominal organs. These results appear simple and conclusive in favour of the toxin obtained being a genuine secretion product of the bacilli. They are, however, open to another interpretation—viz., that these observers were really dealing with toxic elements of the comma bacillus which had been released by autolysis and disintegration of the cells. The death-rate of organisms, even in a young cholera culture, is rapid. Gottschlich and Weigang[6] found that in a two-days' culture of cholera at 37° C. only about 10 per cent. of the bacilli were alive and on the third day at most 1 per cent. It still remains to me doubtful if such poisons are of the same character as those developed in the body in the course of a cholera infection.

Pfeiffer is the most distinguished upholder of the doctrine that the cholera poison is contained in the protoplasm of the cell. Young broth cultures have no marked toxic effect on filtration. On the other hand, carefully killed young agar cultures produce an acute intoxication of the same nature as that produced by living cultures. This has been demonstrated by Pfeiffer, in a most careful series of experiments.[7] There can be little doubt that the primary cholera toxin is of the endocellular type, of an unstable character, and is easily converted into less toxic modifications. Its demonstration, therefore, requires a careful and conservative technique. Subsequent research has not substantially modified the respective standpoints indicated except in so far as Pfeiffer's conclusions have met with the widest acceptance. Since 1896 neither Behring and Ransom nor Metchnikoff and his colleagues have, so far as I am aware, published any further communications and one may assume that the results did not fulfil their expectations. The reader must be referred to the papers by Kraus and Prautschoff[8] upon cholera and other vibrios and their hæmotoxins, toxins, and antitoxins, as the details do not admit of a short analysis.

Whilst the various methods employed have led to the demonstration of toxic elements in fluid cholera cultures the filtrates obtained are less toxic than the unfiltered cultures and have not given satisfactory immunising results. The criterion of an active toxin is the production of a potent antitoxic serum. Judged by this standard the soluble

[6] Gottschlich and Weigang: Zeitschrift für Hygiene, p. 376, vol. xx., 1895.

[7] Pfeiffer, ibid., vol. xi., 1892, p. 393; vol. xv., 1894, p. 268; and vol. xx., 1895, p. 217.

[8] Kraus and Prautschoff: Centralblatt für Bacteriologie, Abtheil i., Hefte 3 and 4, 1906.

toxins hitherto described have proved disappointing. This will be evident by reference to the immunising experiments of Metchnikoff and his co-workers. Two horses received subcutaneous injections of the filtered toxin prepared in the manner described, the initial dose being ten cubic centimetres. At the end of six months the horses were able to tolerate injections of 200 cubic centimetres. The injections were made at intervals of from 10 to 12 days and caused considerable local reaction. The serum mixed with the filtered toxin and injected into guinea-pigs subcutaneously gave the following results. After three months' treatment, and a total injection of 350 cubic centimetres of toxin, three cubic centimetres of the horse's serum neutralised one and a half lethal doses of the filtered toxin. After six months, and the injection of 950 cubic centimetres of toxin, one cubic centimetre of serum neutralised four lethal doses of toxin. The figures indicate that the immunising value of the toxin and the antitoxin was of a feeble character. The Behring-Ransom serum was likewise found to neutralise the same toxin. The serums prepared in goats by Pfeiffer by the injection of the bodies of the bacilli were of high bacteriolytic power but without demonstrable antitoxic action for the intracellular poison. The serum did not protect against the killed and toxic bodies of the bacilli. Whilst, therefore, it is recognised that a cholera serum should possess antitoxic qualities, Pfeiffer's serum did not exhibit these and Metchnikoff's, even after a prolonged period of immunisation, did not in its effects strikingly exceed the range of action of normal serum. Further, the feasibility of preparing an antitoxin for that primary poison which exists as an integral constituent of the cholera bacillus has remained a matter of dispute, and has been regarded as impracticable by many competent bacteriologists.

It was with the object of obtaining a definitive result, either in a positive or negative sense, that the following investigation was undertaken. As I have already briefly stated in a previous paper upon an antitoxic typhoid serum the experiments with the cholera endotoxin were successful.[9] The cold-grinding method adopted gave the necessary conditions for obtaining the cholera endotoxin directly from the living cell and studying its properties. Virulent cultures of the comma bacillus were employed for the purpose of the experiments. The organisms were cultivated on nutrient agar in Roux bottles. A watery emulsion of the eighteen hours'

[9] Macfadyen: Proceedings of the Royal Society, March 8th, 1906; Brit. Med. Jour., April 21st, 1906.

growth was made, which was then washed in a high-speed centrifuge. The separated organisms were triturated at the temperature of liquid air and the product was taken up in 1 in 1000 caustic potash. On spinning, a clear supernatant fluid representing a 10 per cent. extract of the comma bacilli was obtained and was treated very rapidly with chloroform vapour. The cell juices were in every instance sterile and were acutely toxic to experimental animals. The quantitative yield from the various grinds was remarkably constant and averaged about ten milligrammes of solid matter per cubic centimetre of the juices. This being the case it was possible to trace any parallelism that might exist between the virulence and the toxicity of the cultures—a matter of considerable theoretical and practical importance. It may be here stated that the cultures of high virulence yielded the most toxic and the cultures of low virulence the least toxic juices, whilst in those instances in which the virulence had been allowed to diminish to such an extent that, e.g., two platinum loops of a culture did not kill the guinea-pig, the toxicity of the juice suffered a corresponding drop, one-half cubic centimetre and even one cubic centimetre failing to kill, whilst one-tenth cubic centimetre from a virulent culture killed acutely. This leads me to conclude that as regards the cholera endotoxin virulence and toxicity are intimately related, inasmuch as increased virulence implies increased toxicity and *vice versâ*. This observation may explain the discrepant results obtained by different workers and in any case justifies the use I have constantly made of virulent cultures in my investigations upon this and other pathogenic organisms.

The variations in toxicity of the cholera cell juices I consider to have been largely dependent on variations in the toxic quality of the material at the time of grinding. The index for a good yield of endotoxin is the virulence of the given culture and this varies under laboratory conditions despite all precautions taken, and is liable to sudden drops. It is a source of trouble in connexion with experimental work on the cholera organism.

Toxicity of the cell juices.—From the most virulent cultures toxic extracts were obtained which on peritoneal injection killed guinea-pigs acutely in doses of $_{1}^{1}{}_{0}$th and $_{2}^{1}{}_{0}$th cubic centimetre, whilst $_{5}^{1}{}_{0}$th cubic centimetre rendered the animals ill. The average lethal dose for guinea-pigs of 300 grammes weight was $_{1}^{1}{}_{0}$th cubic centimetre (about one milligramme). The extracts from cultures of lower virulence killed in doses of 0·3 and 0·5 cubic centimetre. Where the virulence was feeble doses of 0·5 cubic centimetre and upwards were necessary to kill the animals. The endotoxin also acted sub-

cutaneously. The effects were the same as on peritoneal injection—congestion of the intestine, hyperæmia of the organs, and hæmorrhages in the stomach. The doses killing subcutaneously were two cubic centimetres and one cubic centimetre. In the instances tested 0·5 and 0·2 cubic centimetre failed to kill. The results were not so constant as those obtained with the smaller peritoneal doses.

The endotoxin likewise killed rabbits acutely on intravenous injection. The symptoms were collapse, occurring about three-quarters of an hour after the injection, acute diarrhœa, and a rapid fall of temperature ending in death, at times within one hour. From the most virulent cultures $\frac{1}{20}$th and $\frac{1}{10}$th cubic centimetre of the cell juices killed acutely with the above symptoms. The average lethal dose ranged from 0·3 to 0·5 cubic centimetre. The filtered juices were also toxic—e.g., 0·2 and 0·5 cubic centimetre of a filtered juice proved acutely fatal. The rabbit was more tolerant to subcutaneous injections—e.g., one, two, and three cubic centimetres of the endotoxin did not kill but produced considerable local swelling and induration. The guinea-pig proved more uniformly susceptible and was therefore mainly employed in the subsequent serum tests.

The endotoxin was acutely fatal to goats on intravenous injection. A goat died within 12 hours after an injection of $\frac{1}{10}$th cubic centimetre (about one milligramme). A second goat developed persistent diarrhœa after a dose of $\frac{1}{100}$th cubic centimetre (about $\frac{1}{10}$th milligramme). In three other instances $\frac{1}{100}$th cubic centimetre rendered the animals ill, whilst $\frac{1}{80}$th and $\frac{1}{50}$th cubic centimetre produced acute diarrhœa and death. These examples indicate the potent nature of the cholera endotoxin when introduced into the blood stream of a susceptible animal, $\frac{1}{10}$th milligramme of the material inducing marked toxic effects.

In all the above experiments the freshly prepared endotoxin was injected. The juices deteriorate in toxic power on keeping and are readily modified by heat, as will be seen from the following experiment :—

Toxic Cholera Cell Juice.

Half hour at 55° C.	Half hour at 60° C.	Unheated.
Guinea-pig : 1·0 c.c. alive.	Guinea-pig : 1·0 c.c. alive.	Guinea-pig : 1·0 c.c. dead.
Guinea-pig : 0·5 c.c. alive.	Guinea-pig : 0·5 c.c. alive.	Guinea-pig : 0·5 c.c. dead.
Guinea-pig : 0·3 c.c. alive.	Guinea-pig : 0·3 c.c. alive.	Guinea-pig : 0·3 c.c. dead.

The toxicity of the cell juices in the above doses was destroyed after half an hour's heating to 55° and 60° C. The results were the same in the case of the typhoid endotoxin obtained under similar conditions. The soluble toxins obtained by Ransom and by Metchnikoff resisted heating to 100° C.

Immunising experiments.—These were carried out on the rabbit and the goat. Normal rabbit's and goat's serum had no appreciable neutralising effect on the toxins obtained directly from the cholera organism—one cubic centimetre of normal serum did not neutralise two lethal doses of the endotoxin. The rabbits received subcutaneous injections of the toxic cell juices and proved tolerant to considerable doses. One rabbit received at intervals of a week 1, 2, 3, and 5 cubic centimetres of the toxin subcutaneously. The serum was tested seven days after the last injection. The serum-toxin mixture was kept at 37° C. for half an hour before injection into the guinea-pig. The results are given in the following table :—

Guinea-pig.	Dose.	Result.
1	2 c.c. toxin + 2 c.c. serum	Alive
2	2 c.c. ,, + 1 c.c. ,,	,,
3	2 c.c. ,, + ½ c.c. ,,	,,
4	1 c.c. ,, + 2 c.c. ,,	,,
5	1 c.c. ,, + 1 c.c. ,,	,,
6	1 c.c. ,, + ½ c.c. ,,	,,
7	1 c.c. ,, + 2 c.c. *normal* serum	Dead
8	0·2 toxin	,,

One cubic centimetre of the toxic cell juice contained five ascertained lethal doses. The treated rabbit's serum had therefore acquired distinct anti-endotoxic properties, inasmuch as 0·5 cubic centimetre neutralised ten lethal doses of the endotoxin, whilst one cubic centimetre of normal serum did not neutralise two lethal doses. Other treated rabbits gave similar results after a short period of immunisation—e.g., in two instances tested, $\frac{1}{20}$th cubic centimetre of the serum protected against three and $\frac{1}{30}$th cubic centimetre against two ascertained lethal doses of the endotoxin. The injection of the fresh toxin in a condition favouring absorption no doubt favoured the result. By means of these experiments one was able to demonstrate at the outset the production of an anti-

body for the cholera endotoxin. It was also evident that one was dealing with an active toxin.

The further immunising experiments were carried out on the goat and the intravenous method of injection was employed. This animal is highly susceptible to the action of the cholera endotoxin, as will be seen from the figures already given, and a careful method of dosage is necessary for successful immunisation. A goat received intravenously at intervals of a week $\frac{1}{100}$th, $\frac{1}{50}$th, $\frac{1}{10}$th, $\frac{1}{5}$th, $\frac{1}{20}$th, and $\frac{1}{3}$th cubic centimetre of toxic cell juices. The animal was ill after the first injection and the subsequent doses produced distinct reactions. The serum was tested after the sixth injection. The results are given in the accompanying table :—

Guinea-pig.	Dose.	Result.
1	2 c.c. toxin + 2 c.c. serum.	Alive.
2	2 c.c. ,, + 1 c.c. ,,	Dead.
3	2 c.c. ,, + ½ c.c. ,,	Alive.
4	1 c.c. ,, + 2 c.c. ,,	,,
5	1 c.c. ,, + 1 c.c. ,,	,,
6	1 c.c. ,, + ½ c.c. ,,	,,

The cell juice contained four ascertained lethal doses in one cubic centimetre. The figures therefore show that one-half cubic centimetre of the serum protected against eight lethal doses of the endotoxin. It had been previously ascertained that one cubic centimetre normal goat's serum will not protect against two lethal doses of the poison, nor, as a matter of fact, was there any appreciable action in two cubic centimetres of normal serum.

A second goat was treated more deliberately and for a longer period, beginning with sublethal initial doses of the endotoxin, and tolerance was established to otherwise fatal amounts. The weekly injections made were $\frac{1}{100}$th, $\frac{1}{100}$th, $\frac{1}{50}$th, $\frac{1}{50}$th, $\frac{1}{50}$th, $\frac{1}{30}$th, $\frac{1}{30}$th, $\frac{1}{20}$th, $\frac{1}{20}$th, $\frac{1}{20}$th, $\frac{1}{15}$th, $\frac{1}{15}$th, $\frac{1}{15}$th, $\frac{1}{12}$th, $\frac{1}{12}$th, and $\frac{1}{3}$rd cubic centimetre. The animal was ill after the first injections of $\frac{1}{100}$th, $\frac{1}{30}$th, $\frac{1}{20}$th, and $\frac{1}{15}$th cubic centimetre. The total amount injected was about one cubic centimetre, or 10 milligrammes, of solid matter. The injections extended over four months, the animal at the end of that time being alive and well. In the case of this goat the neutralising power of its serum was always tested against the same amount of toxic cell juice—viz., one cubic centimetre, and containing from three to five

ascertained lethal doses. The serum-toxin mixture was kept at 37° C. for half an hour before injection into the guinea-pig. The following table represents the results obtained.

Serum Tests.

Goat.	Blood sample.	Ascertained lethal doses of toxin.	Amount of serum.	Guinea-pig. Result.
After	6 injections.	3	$\frac{1}{10}$ c.c.	Alive.
,,	,, ,,	3	$\frac{1}{20}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{50}$ c.c.	,,
,,	13 ,,	5	$\frac{1}{10}$ c.c.	,,
,,	,, ,,	5	$\frac{1}{20}$ c.c.	,,
,,	14 ,,	3	$\frac{1}{20}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{20}$ c c.	,,
,,	,, ,,	3	$\frac{1}{50}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{50}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{100}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{100}$ c.c.	,,
,,	15 ,,	3	$\frac{1}{200}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{200}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{400}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{300}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{500}$ c.c.	,,
,,	,, ,,	3	$\frac{1}{500}$ c.c.	,,

The figures demonstrate that marked anti-endotoxic properties had developed in the serum of the goat and had increased to the degree that $\frac{1}{500}$th cubic centimetre of the serum neutralised three ascertained lethal doses of the endotoxin, a property not possessed by one cubic centimetre of normal serum. A few tests were made with the goat's serum on the rabbit and showed that the serum likewise acted on intravenous injection. For example, $\frac{1}{50}$th cubic centimetre of the serum protected a rabbit against the intravenous injection of from three to four lethal doses of the endotoxin, and in other tests similar results were obtained.

The experiments having served their purpose have not in the meanwhile been carried further, although I have little doubt that still higher serum titrates could have been obtained. They demonstrate not only the feasibility of producing an antibody for the cholera endotoxin by the method

adopted, but also the practicability of raising to a marked degree the anti-endotoxic value of the serum. It remains to add that the goat's serum likewise possessed agglutinative and bacteriolytic properties—e.g., $\frac{1}{2000}$th cubic centimetre of the serum agglutinated the cholera bacilli and $\frac{1}{1000}$th cubic centimetre gave Pfeiffer's reaction. The experiments in this direction were not carried further than was necessary to demonstrate the existence of these bodies.

Conclusions.

The experiments show:—1. That acutely toxic cell juices possessing active immunising properties can be obtained from the cholera organism by the method employed. 2. That an antibody can be produced for that primary poison which exists as an integral constituent of the cholera bacillus—a matter upon which there have been considerable dispute and difference of opinion. 3. That the anti-endotoxic power of the serum can be raised to a marked degree—a matter of equal importance. 4. That the serum in addition to its anti-endotoxic possessed agglutinative and bacteriolytic properties. 5. That in the case of the cholera organism there exists an intimate relationship between its virulence and toxicity. 6. That the cholera endotoxin obtained under the conditions described is thermolabile, being readily destroyed at 55° and 60° C.

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